PhD Thesis

Iron uptake mechanism of chloroplasts

Brigitta Lantos

Supervisors:

Dr. Ádám Solti and **Dr. habil. Ferenc Fodor**

PhD Programme in Biology (Head of Programme: Prof. Anna Erdei)

Experimental Plant Biology Programme (Head of Programme: Dr. Gábor M. Kovács)

Department of Plant Physiology and Molecular Plant Biology, Institute of Biology, Faculty of Science, Eötvös Loránd University

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Introduction

Iron is essential micronutrient in plants required by fundamental metabolic processes. By its redox properties iron is a particularly abundant element in cofactors of enzymes, such as photosynthetic and mitochondrial electron transport chain components. Consequently, role of iron in plant growth and development is unequivocal. The extremely high demand for iron of chloroplast-associated processes – such as photosynthesis and chlorophyll biosynthesis – indicate the importance of chloroplasts in iron homeostasis of shoot tissues. In the absence of iron, the biosynthesis of essential cofactors such as hem groups and Fe-S clusters is strongly disturbed, which results in the retardation of the assembly of thylakoid complexes and thus the dysfunction of photosynthetic electron transport and enzyme reactions. In mesophyll cells, at least 80% of shoot iron content is localized in chloroplasts. Despite its importance, deficiency of iron is a common and serious problem in agricultural practices leading to chlorosis, decrease in the biomass production and crop yield or even to the death of plants as a long-term effect. Thus, the relevance of the mechanism of iron utilisation of chloroplasts is a process of prime importance.

Although iron is necessary in plants for proper functioning it can be toxic as free ferrous form. Thus iron is complexed by different organic ligands immediately after taken up by plants. To avoid both iron deficiency and toxicity symptoms iron uptake and transport is strictly regulated in plants. Despite its importance only a few pieces of information are known on the transport mechanisms between the cytoplasm and chloroplasts including uptake of iron into the stroma.

Among angiosperm plant species two different iron transport strategies were found so far. These iron uptake systems exist in different organs, tissues, taxa and development stages (Verbon *et al*., 2017). Dicots and the majority of monocots use a reduction-based mechanism (Strategy I) to take up iron from the soil medium (Römheld, 1987). The key component of this strategy are Ferric Reductase Oxidase (FRO) enzymes in the rhizodermis since it reduces ferric complexes and, subsequently, the released Fe^{2+} can be transported into the cytoplasm of rhizodermis cells. Strategy II is a chelation-based mechanism. Although the chelation-based iron transport strategy exists in all higher plants at tissue level, members of the Poaceae family especially evolved to take up iron by their roots based on this strategy. This strategy relies on the exudation of phytosiderophores, which solubilize and bind Fe(III) and the plants take up Fe(III)-phytosyderophore complexes by YSL transporters (Curie *et al*., 2009).

Although iron is essential for proper functioning of vital processes free ferrous form can also be toxic as it can catalyse Fenton reaction in the presence of hydrogen peroxide inducing the formation of reactive oxygen species that leads to oxidative damage in proteins and lipids (Ravet and Pilon, 2013). In order to avoid these effects iron is bound by chelators and transported in a form of Fe(III)-complexes (Curie *et al.*, 2009; Haydon and Cobbett, 2007). In xylem exudates iron is present primarily as $Fe(III)_3$-$(citrate)_3$ complex (Rellán-Álvarez *et al*., 2010). Nicotianamine (NA) is a ubiquitous non-proteinogenic amino acid and belongs to the potential complexing molecules of metal ions (von Wirén *et al.*, 1999, Ariga *et al*., 2014). Although several ligands were detected in plants which can bind iron, direct evidences are not available on the chemical forms of iron in the cytoplasm of mesophyll cells (Bashir *et al*., 2016), from where iron is taken up into the chloroplasts. Thus, ***in vivo*** **substrate(s) of the chloroplast uptake machinery are still not known.** We intended to clarify the nature and iron to ligand stoichiometry of the substrate of iron acquisition machinery.

Chloroplasts are semiautonomous organelles of the plant cells bordered by two envelope membranes. In the outer envelope numerous *β*-barrel type proteins (OEPs) were described which supposedly have a role in the iron transport, however, no specific iron transport proteins were identified using direct methods (López-Millán *et al*., 2016; Solti *et al*., 2016; Vigani *et al*., 2019). Outer envelope proteins and protein complexes mainly have prokaryotic origin (Block *et al*., 2007). Gram negative cyanobacteria – evolutionary ancestors of chloroplasts – are able to take up iron in both Fe(III)-chelate and Fe(III)-oxide forms. Additionally, a voltage-dependent step was observed in Fe(III)-uptake mechanism of the plasmalemma (Braun, 2003). Although membrane potential contributes to the iron uptake in prokaryotic organisms, **the role of the electrochemical membrane potential gradient in the function of chloroplast iron uptake system is still not clear.** Thus, we induced alterations in the membrane potential to provoke affects in the chloroplast iron uptake.

Only few pieces of information are available on the iron uptake through the inner envelope of chloroplasts. In *Arabidopsis*, FRO7 is a crucial participant in chloroplast iron acquisition as this enzyme is responsible for the reduction of Fe(III)-chelates (Jeong *et al*., 2008). Subsequently, the reduced Fe^{2+} can be taken up by the suggested PIC1-NiCo complex (Duy *et al*., 2007; Duy *et al*., 2011). Although some other chloroplast iron uptake and homeostasis related components were identified so far, the function and mechanism of action of these proteins have not been studied yet. Components of the reduction-based strategy in the

root plasma membrane are strongly upregulated/expressed under iron deficiency while iron excess has a strong negative feedback on both *Fro2* and *Irt1*. **In contrast to the root iron uptake, hardly any pieces of information are available on the regulation of chloroplast iron uptake machinery components (FRO7, PIC1) under low as well as excess iron nutrition.** Thus, we investigated the effect of altered iron nutrition to the ferric chelate reductase activity of chloroplast inner envelope membranes and the iron uptake of chloroplasts isolated from plants grown under iron deficient and iron excess conditions.

Materials and Methods

Oilseed rape (*Brassica napus* L. cv. DK Exquisite) and **sugar beet** (*Beta vulgaris* L. cv. Orbis) plants growing in hydroponics on ¼ and ½ Hoagland solution, respectively, were subjected to the isolation of intact chloroplasts and inner envelope vesicles. The model taxa were chosen since chloroplast Fe uptake studies require a large amount of isolated chloroplasts.

To provide optimal **iron supply** 20 μM Fe(III)-EDTA was applied in the nutrient solution. In order to obtain well-developed Fe chlorosis and supraoptimal iron supply, another group of seedlings were precultivated up to four-leaf stage in the same nutrient solution and then transferred to iron-free nutrient solution also containing 0,5% (w/v) $CaCO_3$ or 100 μM Fe, respectively. Leaves that emerged during the next 3 weeks were used for chloroplast isolation from both Fe-sufficient and Fe-deficient plants.

Substrate-preference of chloroplast iron uptake mechanism as well as the effect of divalent transition metal cations and oxoanions on the outer envelope process was studied on intact chloroplasts. **Intact chloroplasts** were isolated and purified on a stepwise sucrose gradient. To detect mitochondrial contamination and estimate the integrity of the chloroplasts, western blots were carried out.

Iron uptake was assessed from the total chloroplast Fe content before and after a 30 min Fe uptake assay. Total chloroplast Fe was measured with bathophenantroline disulphonate (BPDS) according to Solti *et al*. (2012).

To study the role of potential gradient in uptake mechanism, iron uptake activity of chloroplast was measured in the presence of cations and oxoanions as well as **DCMU**

(electron transport inhibitor) and **CCCP** (ionophore) at 100 μM Fe(III)-citrate (Fe:citrate 1:1) Fe source.

Fe–nicotianamine (NA) complexes and Fe(III)-citrate used in uptake assay were characterised by Mössbauer spectroscopy. The element content of chloroplasts was measured before and after the uptake assay.

The FCR activity of inner envelope was investigated on **isolated envelope vesicles**. After isolating intact chloroplast membranes were broken and the different membranes (outer and inner envelope and thylakoids) were separated on a stepwise sucrose gradient by ultracentrifugation. To control the **identity and purity of isolated vesicles,** immunoblots were performed against main marker proteins: AOX 1/2 for mitochondrial contamination, TOC75 and TPT as for chloroplast outer and inner envelope markers, respectively, and apoLHCII as for thylakoid contamination marker. NADPH and FAD was enclosed in the inside of the isolated r*ight-side-out* vesicles. The reaction was initiated by adding Fe(III)-EDTA to the coenzyme-containing envelop vesicle fraction. The reducing activity of the envelope was determined with BPDS method based on the amount of Fe^{2+} resulted in the enzyme reaction.

Results and conclusions

I. Substrate preference of the iron uptake mechanism of chloroplasts

Chloroplasts more effectively utilize Fe(III)–citrate 1:1 compared to the other Fe-complexes potentially can be present in the cytoplasm such as Fe(III)-citrate 1:10 and Fe(III)-malate. Although Fe(III)-malate was transferred on the outer envelope more effectively than the Fe(III)-citrate, uptake system of the inner envelope was not able to use it as a substrate. The uptake of Fe(III)-malate can be facilitated by adding citrate to the uptake medium which can be explained by a ligand-exchange or the formation of a new mixed complex including both organic acids as a ligand. Furthermore iron uptake of chloroplasts was significantly lower in the presence of Fe(II)-NA and Fe(III)-NA complexes. Chloroplasts were able to take up iron from neither Fe(III)-EDTA nor Fe(III)-EDDHA. **In conclusion, the *in vivo* substrate of the chloroplast iron uptake mechanism is most probably the Fe(III)-citrate 1:1.**

II. Effect of divalent cations, oxoanions and the membrane potential uncoupling ionophore on the iron uptake of chloroplasts

The presence of inorganic salts did not affect chloroplast integrity and had no effect on the Fe forms incorporated into chloroplasts as judged by Mössbauer spectroscopy.

Iron uptake in intact chloroplasts was enhanced by transition metal cations, whereas it was hampered by oxoanions. The chloroplast Fe uptake was also affected by the quality (Zn^{2+}>Cd^{2+}>Mn^{2+}) and the concentration (200 μM>500 μM) of transition metal cations in the uptake medium. In addition, the effect of cations and anions was similar in the absence and presence of DCMU although the iron uptake activity decreased significantly after adding DCMU. It is because of the limited NADPH production since NADPH is required by iron uptake mechanism of chloroplasts. It was also shown that CCCP completely blocked the iron-uptake processes in chloroplasts. Taken together the inhibitory effects of DCMU on the Fe movement across the IE membrane and the similar influence of transition metal cations and oxoanions on the DCMU-sensitive and insensitive Fe uptake, necessarily, **an additional regulatory role of chloroplast outer envelope in Fe uptake, is strongly supported.**

III. The effect of iron supply on the iron uptake of chloroplasts

Iron content of intact chloroplasts used in the experiment was 10-times higher in plants growing on optimal Fe-concentration than in iron deficient plants while **it was not changed significantly in plants with supraoptimal Fe-supply.**

Iron uptake activity of intact chloroplasts isolated from plants grown with different iron-supply was measured in the presence of Fe(III)-citrate at increasing iron concentration range. Fe-uptake of iron deficient chloroplasts decreased considerably compared to optimal iron-containing chloroplasts, however the ratio of iron content of chloroplasts before and after the uptake assay (50% of initial iron content was taken up) was not changed significantly.

The iron uptake of chloroplasts from plants grown with supraoptimal iron-supply was also lower compared to that of the chloroplasts with optimal iron-supply similarly to the negative feedback on the iron content of the chloroplasts.

Iron uptake measurement using chloroplasts of different iron-supply indicate that **iron uptake system of chloroplasts is regulated depending on the iron supply.**

IV. The effect of iron-supply on the activity of chloroplast envelope ferric chelate reductase enzyme

Ferric chelate reductase (FCR) assays proved that **the FCR activity was not affected by iron deficiency**, even though iron uptake of intact chloroplasts was decreased. Based on this result we conclude that the efficiency of cFRO enzyme is nearly optimal, on the other hand, **iron uptake of chloroplasts was limited by the smaller size of the iron deficient chloroplasts and the reduced amount of the available NADPH. Supraoptimal iron-supply resulted in a decrease in the affinity of ferric chelate reductase enzyme** and also in the iron content of the chloroplast and iron uptake activity of intact chloroplasts which may be caused by the reduced expression of *Fro7* and/or other posttranscriptional-posttranslational modification avoiding the effect of toxic iron.

Main conclusions:

a) Based on the DCMU-insensitive and uncoupling ionophore (CCCP)-sensitive stimulating effects of transition metal cations and inhibitory effects of oxoanions on the Fe uptake of intact chloroplasts we propose that **a voltage-dependent Fe(III)-complex transport system is involved in the Fe(III)-citrate transport across the chloroplast outer envelope**.

b) According to our substrate preference assays on chloroplast iron uptake machinery we propose that **Fe(III)–citrate 1:1 is the *in vivo* substrate for FRO enzyme essential in the iron uptake from cytoplasm to the chloroplast stroma.**

c) Iron uptake activity of intact chloroplasts in parallel with the kinetics of the inner envelope associated FRO enzyme indicate that the **supraoptimal iron reduced while iron deficiency did not change the reduction capacity of it**.

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Publications

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